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Development of the proepicardial organ in the zebrafish

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Abstract

The epicardium is the last layer of the vertebrate heart to form, surrounding the heart muscle during embryogenesis and providing signaling cues essential to the continued growth and differentiation of the heart. This outer layer of the heart develops from a transient structure, the proepicardial organ (PEO). Despite its essential roles, the early signals required for the formation of the PEO and the epicardium remain poorly understood. The molecular markers *wt1* and *tcf21* are used to identify the epicardial layer in the zebrafish heart, to trace its development and to determine genes required for its normal development. Disruption of lateral plate mesoderm (LPM) migration through knockdown of *miles apart* or *casanova* leads to cardia bifida with each bilateral heart associated with its own PEO, suggesting that the earliest progenitors of the epicardium lie in the LPM. Using a gene knockdown approach, a genetic framework for PEO development is outlined. The *pandora/spt6* gene is required for multiple cardiac lineages, the zinc-finger transcription factor *wt1* is required for the epicardial lineage only and finally, the cell polarity genes *heart and soul* and *nagie oko* are required for proper PEO morphogenesis.

Keywords: Epicardium; Zebrafish; Proepicardial organ; *wt1*; *tcf21*; Cell polarity; *spt6*

Introduction

The heart is one of the first organs to form during vertebrate development. The primitive heart tube consists of two layers, an inner endocardium, continuous with the vasculature, and the surrounding myocardial layer (Fishman and Chien, 1997). Lineage analyses demonstrate that heart tube progenitors are located in the lateral plate mesoderm (DeHaan, 1965; Serbedzija et al., 1998). Progenitor cells migrate toward the midline to form the two-layered heart tube and subsequent morphological changes are necessary to shape this primitive heart tube into a complex multi-chambered organ. Valves develop between chambers helping to provide directional flow, the myocardial layer thickens and becomes trabeculated and a coronary vasculature is established to supply cardiac muscle with oxygenated blood. These changes are dependent upon a third outer layer of the heart to form, the epicardium.

The epicardium is a mesodermally derived cell layer that covers the myocardial layer and provides signaling cues essential to the proper development of the heart. The continued growth and differentiation of the myocardium are dependent upon the epicardium (Manner, 1993). Genetic evidence for this requirement was first observed in mice carrying targeted deletions of the VCAM-1, α4 integrin and Wilms' Tumor suppressor-1 (WT1) genes. Each of these three genes is essential for the formation of an intact epicardium and null mice die as a consequence of abnormal cardiac development (Kreidberg et al., 1993; Kwee et al., 1995; Moore et al., 1999; Yang et al., 1995). In addition to its role in sustaining myocyte proliferation, the epicardium contains progenitors of the coronary vasculature. Cell lineage tracing analyses using lipophilic dyes and retroviral marking demonstrate that the PEO contains progenitors of the coronary vasculature including endothelial cells and smooth muscle cells (Mikawa and Fischman, 1992; Mikawa and Gourdie, 1996). Preventing epicardium–myocardium interactions leads to coronary vascular defects (Gittenberger-de Groot et al., 2000).

Despite recent studies indicating the essential nature of the epicardium, the signals involved in the formation of the epicardium and its earliest manifestation, the proepicardial organ, remain poorly understood. The earliest location of the epicardial

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precursors is not firmly established. In the chick and mouse, the epicardium originates from the proepicardial organ (PEO), an outgrowth of the septum transversum (Ho and Shimada, 1978; Manasek, 1969; Viragh et al., 1993). The PEO is first visible in close proximity to the sinoatrial pole and atrioventricular junction. PEO cells first contact the dorsal surface of the myocardium at the site of the future atria around stage 17 in birds and 10.5 days postcoitum (dpc) in mice (Hiruma and Hirakow, 1989; Viragh and Challice, 1981; Viragh et al., 1993).

Early cardiac development has been well documented in the zebrafish model system and genetic screens have identified mutations that affect distinct steps in the development of the heart (Stainier, 2001). The development of the epicardial layer has not been characterized. Here, I describe the development of the PEO and the epicardium in the zebrafish using *wt1* and *tcf21/epicardin* as molecular probes and use a gene knockdown approach to establish a hierarchy of genes required for distinct steps of PEO formation.

Materials and methods

Fish stocks

Wild-type zebrafish lines (AB and Tuebingen strains) were used throughout this study. Zebrafish were raised and maintained at the Novartis Institutes for Biomedical Research in accordance with approved animal procedures and protocols. Embryos were kept in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) at 28.5 °C and staged according to or hours post-fertilization (hpf) or days post-fertilization (dpf) at 28.5 °C (Kimmel et al., 1995).

Cloning of zebrafish tcf21/epicardin

A putative zebrafish ortholog of the *tcf21* gene was identified in the zebrafish genomic assembly (Sanger Centre, UK, Accession number NM_001037681). A cDNA fragment was amplified using reverse transcriptase-polymerase chain reaction (RT-PCR) with 24 hpf RNA as the template and gene specific primers: tcf21+: CTCCACGTCCAGTCAGAGAACCTC and tcf21−: TATTTGTTTTATTTACAATTATTC. The fragment was cloned into the pCRII-TOPO vector (Invitrogen). *Eco*RV digestion and SP6 RNA polymerase were used to make DIG labeled antisense probe.

In situ hybridization and antibody staining

In situ hybridizations with the *wt1* probe were performed as previously described (Serluca and Fishman, 2001). The probe was linearized with *Eco*RI and antisense transcript synthesized using T7 RNA Polymerase. For double labeling using the *wt1* RNA probe and myosin heavy chain protein, embryos were processed for whole-mount in situ hybridization, rinsed in phosphate buffered saline with 0.1% Tween-20 (PBT) and then probed with the MF20 monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa). Briefly, stained embryos were incubated with a 1:100 dilution of MF20 in 5% normal goat serum (NGS)/PBT for 2 h, washed over 2 h with PBT, then probed with an anti-mouse-POD secondary antibody (Roche Applied Science, Indianapolis, IN) and the color developed using 3,3′ diaminobenzidine.

Morpholino injections

Morpholino oligos (MO) were obtained from Gene-Tools (Philomath, OR) and diluted to 1 mM using Danieau Buffer (5 mM HEPES, pH 7.1, 58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$). The morpholino sequences, the concentrations used for injections and the approximate amount injected are described below: *mil*MO (5′-AGACGGCAAGTAGTCATTCAGAGGG-3′), 250 μM, 9 ng; *cas*MO (5′-CAGGGAGCATCCGGTCGTCGAGATACAT-3′) 150 μM, 6 ng; *has*MO (5′-TGTCCCGCAGCGTGGGCATTATGGA-3′), 100 μM, 4 ng; *nok*MO (5′-TGAGGTCAGCAGCGGCTCCAAACAC-3′), 250 μM, 9 ng; *heg*MO (5′-GTAATCGTACTTGCAGCAGGTGACA-3′), 100 μM, 4 ng; *sih*MO (5′-CATGTTTGCTCTGATCTGACACGA-3′), 100 μM, 4 ng; *spt6*MO (5′-CCTCGCTCTCGATGAAGTCAGACAT-3′), 100 μM, 4 ng. The morpholino sequences for *casanova*, *silent heart*, *pandora/spt6*, *heart of glass*, *heart and soul/aPKC* and *nagie oko* have been previously described (Dickmeis et al., 2001; Keegan et al., 2002; Mably et al., 2003; Rohr et al., 2006; Sehnert et al., 2002). Danieau's buffer was used as a control in morpholino injection experiments.

The *wt1* morpholino (5′-GAGCAAGAGATACTGACCTGAAGGC-3′) was designed to a splice donor site and injected at a concentration of 250 μM (approximately 9 ng). RNA from a pool of injected embryos and mock-injected controls was obtained using the RNeasy kit (Qiagen Inc., Valencia, CA) following the manufacturer's specifications. The One Step RT-PCR kit (Qiagen Inc., Valencia, CA) was then used to amplify the *wt1* coding sequence using the following primers: WT1FWD: 5′-CAAATGGCGTCACAGTTGGAGTGC-3′, WT1REV: 5′-GTGCATCTGTAAGTGCGACGTTTA-3′.

Histological methods

Wild-type or *wt1*MO embryos were fixed in 4% PFA overnight at 4 °C, dehydrated through an ethanol series (50%, 75%, 95%, 100%) before being set in JB-4 plastic blocks according to the manufacturer's instructions (Polysciences Inc., Warrington, PA). Three to four micron sections were cut and stained with hematoxylin and eosin. Embryos sectioned following RNA whole-mount in situ hybridization were fixed in 4% formaldehyde for 30 min at room temperature and then rinsed several times in PBT before being mounted into JB-4 blocks as described above. Sections between 6 and 8 μm in thickness were taken using a Leica microtome. Images were obtained using a Nikon Eclipse 80i microscope and a Nikon DS-5Mc camera.

Results

The epicardium is present in the embryonic zebrafish heart

The epicardial layer that surrounds the myocardium is the last layer of the heart to form and contains progenitors of the coronary vasculature. Previous anatomical analyses of the zebrafish adult heart have confirmed the presence of both the epicardium as well as a coronary vasculature (Hu et al., 2000). The origin and timing of their development have not been described. I use here both histological methods and molecular markers to test whether the epicardium and the PEO are present in the zebrafish embryo.

Analysis of sections taken of 4 dpf zebrafish embryos reveals a third cell layer that surrounds the myocardium, the embryonic epicardium (Fig. 1A). Nuclei of the epicardium appear more ovoid and can be distinguished from the rounder myocardium nuclei. The Wilms' Tumor suppressor gene (*WT1*) is a zinc-finger transcription factor expressed in the epicardium in mammals and birds (Armstrong et al., 1993; Carmona et al., 2001; Pritchard-Jones et al., 1990). Whole-mount in situ hybridization with the zebrafish homolog *wt1* using 4 dpf embryos, a stage where the epicardium is visible by histology, demonstrates that cardiac *wt1* expression is strikingly conserved among the vertebrate species. Expression of the transcription factor is seen surrounding the heart muscle at 4 dpf (Fig. 1B). Sectioning of the stained embryos confirms that its expression is confined to the outer epicardial layer (Fig. 1C).

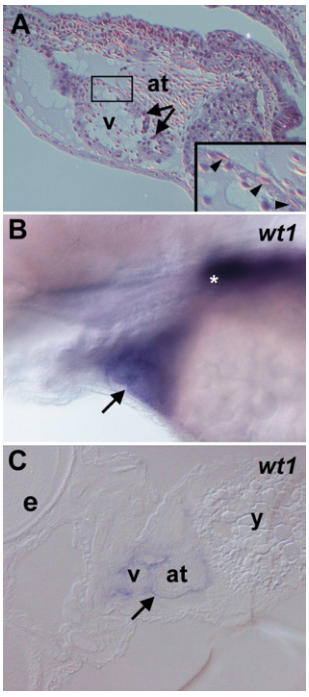

Fig. 1. The epicardium in the larval zebrafish heart expresses *wt1*. (A) Hematoxylin and eosin-stained section of a 4 dpf zebrafish embryo detailing the structure of the embryonic heart. The atrium (at) has a thinner wall than the ventricle (v) and valves are present between the two chambers (arrows). Myocardial nuclei are round while epicardial nuclei (arrowheads in the inset) appear elongated. (B) Whole-mount in situ hybridization of a 4 dpf embryo with the *wt1* probe. The pronephric expression is marked with an asterisk (*) and cardiac staining (arrow) is clearly visible. (C) Frontal section of a 4 dpf *wt1*-stained embryo confirming that the *wt1* expression is localized to the outer cardiac layer (arrow). Anterior is to the left in all panels. e: eye, y: yolk.

wt1 and tcf21/epicardin mark the proepicardial organ

The generation of the epicardium, an epithelial layer, from the PEO is a very dynamic process. In other species, the PEO has been described as having a "cluster of grapes" appearance (Mikawa and Gourdie, 1996; Viragh and Challice, 1981; Viragh et al., 1993). I sought to determine if the zebrafish epicardium develops in a similar fashion to other vertebrates; that is, from a PEO structure. Observations of zebrafish hearts at 50 hpf indicate that clusters of spherical cells distinct from the beating myocardium, are found in close apposition to the heart (Fig. 2 and Supplemental Movies 1 and 2). Clusters can be observed near the sinus venosus, between the atrium and the yolk and near the atrioventricular junction, in contact with the ventricular surface of the heart. The shape of the cells and their clustering bear a striking resemblance to the PEOs of avian and mouse embryos.

To determine if the cell cluster is in fact the PEO, I used *wt1* as a molecular marker to confirm the PEO identity and to trace its development. Cardiac expression of *wt1* is first seen at 38 to 40 hpf (Fig. 3A). At 48 hpf, when the PEO becomes visible, *wt1*-positive cells are generally located at the level of the sinus venosus or adjacent to the atrioventricular junction thus confirming the identity of the cluster as the PEO (Figs. 3B, C, D). Embryos at this stage can have expression in both these areas as seen in Figs. 3B and D or just one of the two. At 3 dpf, *wt1* expressing cells have begun to cover the myocardium along the dorsal surface of the heart. The close apposition of the *wt1*-positive cells with the heart muscle can be visualized in sections of 72 hpf embryos (Fig. 3E). By 5 dpf, the myocardium is covered by *wt1*-positive cells (Fig. 3F, arrow).

tcf21/*epicardin* encodes a basic helix–loop–helix transcription factor expressed in the epicardium and the PEO of the developing mouse embryos (Robb et al., 1998). A cDNA predicted to encode a zebrafish homolog of *tcf21* was cloned by RT-PCR. Cardiac expression of *tcf21* mirrors that of *wt1* providing further confirmation of the PEO identity. Expression of *tcf21* can first be detected by whole-mount in situ hybridization at 40 hpf in a pattern identical to that of *wt1* (Figs. 3G, H). These cells begin to cover the heart by day 3 of development and are found to surround the heart by 96 hpf in an identical fashion to the *wt1*-positive cells (Fig. 3I). Sectioning of the 96 hpf embryos indicates that the *tcf21*-positive layer, like the *wt1*-positive cells, surrounds the myocardium (Fig. 3J).

wt1 is required for the epicardial lineage

Mice homozygous for a null mutation in *WT1* develop cardiac abnormalities and die *in utero* between embryonic days 13 and 15 (Kreidberg et al., 1993). The epicardium fails to form properly and patches of epicardial tissue are only detected in the caudal end of the ventricles (Moore et al., 1999). The expression of the Wilms' Tumor suppressor gene in both the kidney podocytes and the epicardial lineage appears conserved in mammals and teleosts. To test whether function is also conserved, I used a morpholino antisense oligonucleotide (MO) to the *wt1* gene to attenuate gene function by inhibiting proper splicing and assaying the presence of the PEO by *tcf21* expression. The splicing defect in the *wt1* messenger RNA was

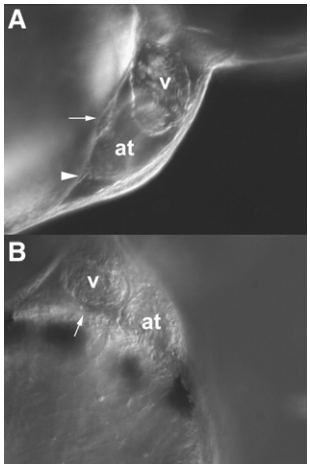

Fig. 2. Proepicardial organ lies in apposition to the myocardium of the embryonic heart. (A) A lateral view of the zebrafish heart at about 50 hpf with clusters of spherical cells seen using differential interference contrast optics nestled between the atrium and the yolk (arrow) and adjacent to the sinus venosus (arrowhead). Anterior is to the right. (B) Ventral view of the heart (50 hpf) with a cluster of cells attached to the outer ventricular wall (arrow). Anterior is at the top of the panel. at: atrium, v: ventricle. Movies of these embryos can be found in the supplemental information section.

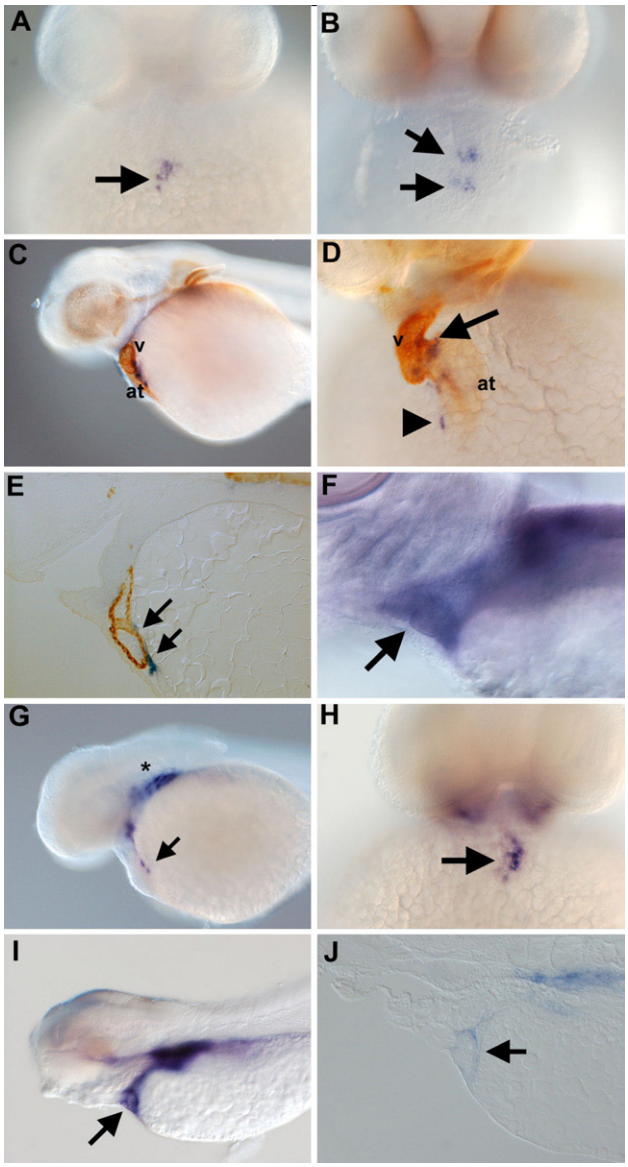

Fig. 3. Expression of *wt1* and *tcf21* in the proepicardial organ. (A) Face view of a 40 hpf embryos stained with *wt1*. The arrow indicates the *wt1*-positive proepicardial organ. (B) At 48 hpf *wt1* staining is visible as clusters (arrows). (C and D) Lateral and face views of whole-mount embryos co-stained with *wt1* (blue) and the anti-myosin heavy chain antibody MF20 (brown). The *wt1* clusters localize to the either the atrioventricular junction (arrow in panel D) or the sinus venosus (arrowhead in panel D). (E) Section of 72 hpf embryo stained with *wt1* (blue) to label the developing epicardium and MF20 (brown) to label the heart muscle. A streak of *wt1*-positive cells is visible along the dorsal aspect of the heart (arrows). (F) At 120 hpf, the *wt1*-positive epicardium surrounds the heart (arrow). (G) Lateral and (H) face views of a 40 hpf embryo stained for *tcf21* expression. *tcf21* transcripts are located in a cluster of cells between the myocardium and yolk (arrows) in an identical pattern to *wt1*. Strong expression of *tcf21* is also visible in the developing arches (asterisk in panel G). (I) At 96 hpf, the heart is completely surrounded by *tcf21*-positive cells (arrow). (J) Sagittal section of a *tcf21*-stained embryo. As with *wt1*, the *tcf21*-positive layer at 96 hpf is confined to the outer epicardial layer (arrow). at: atrium, v: ventricle.

confirmed by RT-PCR analysis (Supplemental Fig. 1). Embryos injected with a 250 μM solution of the *wt1*MO appear morphologically normal at 48 hpf (Figs. 4A, B) although a slight pericardial edema is present in some embryos. The heart is fully

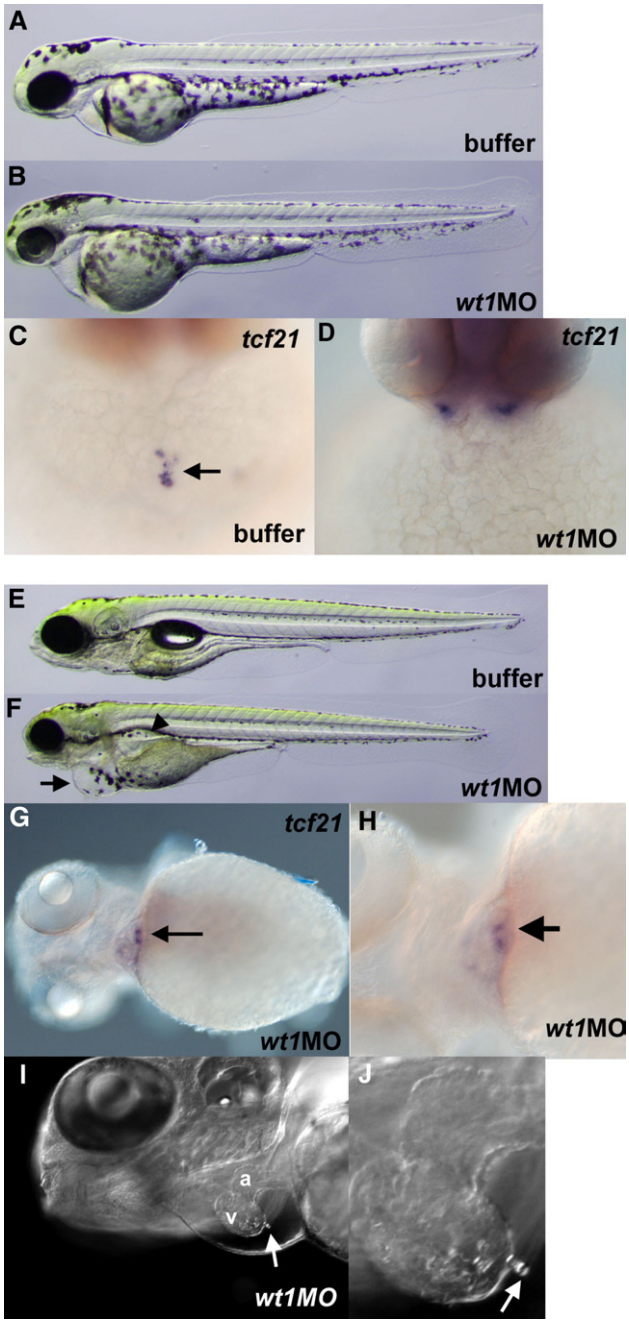

Fig. 4. *wt1* is required for development of the PEO. Live 48 hpf embryos injected with buffer (A) as a control or with *wt1*MO (B). A slight pericardial edema is observed in the *wt1*MO embryos. *tcf21* staining at 48 hpf of (C) buffer-injected or (D) *wt1*MO-injected embryos. The PEO is clearly visible in the control embryos (arrow) but is completely lacking in *wt1*MO embryos (127/127, 100%). (E) Control and (F) *wt1*MO-injected embryos shown at 4 dpf. *wt1*MO embryos display pericardial edema (arrow in panel F) as well as edema in the pronephros (arrowhead). (G) At 4 dpf, a fraction of the embryos (22/68, 32%) have *tcf21*-positive cells adjacent to the heart (arrow). (H) Same embryo as panel G but shown at higher magnification with the *wt1*-positive cells (arrow) nestled between the heart and yolk as observed in 48 hpf wild-type embryos. (I) Live view of a 4 dpf *wt1*MO embryo using DIC optics. The ventricle (v) and atrium (a) are marked and an arrow points to a PEO-like cluster on the surface of the ventricular myocardium. (J) Higher magnification view of the embryo in panel I.

functional, circulation appears normal and ventricular and atrial chambers are clearly demarcated. Staining with *tcf21*, however, reveals a complete lack of the PEO (Figs. 4C, D) indicating that *wt1* acts early in the pathway controlling epicardium development and is required to specify the PEO. When *wt1*MO embryos are examined at 4 dpf, pericardial edema is clearly observed as well as edema in the coelom possibly due to glomerular defects (Figs. 4E, F). While the heart is stretched to a variable extent in *wt1*MO embryos, the heart chambers form normally and circulation appears normal (Supplemental Fig. 2 and Supplemental Movies 3 and 4). Examples of *wt1*MO embryos displaying variable stretching of the heart can be seen in Supplemental Movies 3 and 4. Staining at day 4 reveals that a fraction of *wt1*MO embryos have *tcf21*-positive cells adjacent to the heart (Figs. 4G, H). These cells do not cover the heart and the *tcf21* staining pattern is similar to that seen in wild-type embryos at 48 hpf. Examination of these embryos using DIC optics indicates that PEO-like clusters are present on the myocardial surface of the heart (Figs. 4I, J and Supplemental Movie 5). Thus, the PEO and epicardium in zebrafish share molecular marker expression and a requirement for *wt1* gene function with mammals.

The presence of these *wt1*-positive cells in 4dpf *wt1*MO embryos may be due to the dilution of the *wt1* morpholino as cells divide throughout development. However, quantification of wild-type *wt1* RNA levels relative to beta-actin reveals a similar level of morpholino knockdown efficiency at 2 dpf and 4 dpf and does not support this hypothesis (Supplemental Fig. 3). It is possible that *wt1* is required only for the proper timing of PEO development or that patches of epicardial tissue form even in the absence of *wt1* function as they do in *WT1* null mice.

Cardia bifida embryos display two PEOs

The primitive heart tube forms by the midline migration of precursor cells located in the lateral plate mesoderm (LPM). Large-scale genetic screens in zebrafish have identified a number of mutants that display bifid hearts (Stainier et al., 1996). Analysis of these mutants reveals an underlying defect in the midline migration of the lateral plate mesoderm (Kikuchi et al., 2001; Kupperman et al., 2000; Trinh and Stainier, 2004). In cardia bifida mutants, the myocardial and endocardial progenitors in the LPM continue their developmental and differentiation programs in their original lateral position to yield two beating hearts. In such cases it is not known whether each heart would develop its own epicardium. As most cardiac mutants are either lethal or severely edematous by the stage the epicardium is fully formed, I confined my analysis to the formation of the PEO.

To test whether the PEO, like other progenitors of the heart, originates in the LPM, I used *miles apart* (*mil*) as a model for cardia bifida, inducing the phenotype by morpholino mediated knockdown. *mil* encodes a sphingosine-1-phosphate receptor required for the midline migration of both myocardial and endocardial progenitors (Kupperman et al., 2000). *mil*MO embryos fully recapitulate the mutant phenotype. Staining with *wt1* to mark the PEO and MF20 to mark cardiac muscle at 48 hpf reveals that each of the hearts in *mil*MO embryos is associated with its own PEO (Figs. 5A, B). Knockdown of *casanova* (*cas*), a second gene that when mutated causes cardia bifida, yields a similar result (Figs. 5C, D). The PEO phenotype in *cas*MO embryos appears more severe than in *mil*MO embryos. The presumptive pro-epicardial cells expressing *wt1* are not in close proximity to the bifid hearts and fail to reach their more ventral position near the heart(s). Instead, they are found just ventral and anterior to the first somite. The area that is *wt1*-positive is also larger in *cas*MO embryos than *mil*MO embryos. Despite this difference, the presence of bilateral clusters of *wt1*-positive cells suggests that the origin of the epicardium may also lie in the lateral plate mesoderm.

pandora/spt6 is required for multiple cardiac lineages

The mutant *pandora* develops a much smaller heart with a marked deficiency in ventricular tissue (Stainier et al., 1996). Previous studies have demonstrated that *pandora* mutants fail to properly express both *cardiac myosin light chain 2* (*cmlc2*) and *ventricular myosin heavy chain* (*vmhc*) within the heart field indicating a possible scarceness of competent heart precursors within the LPM (Yelon et al., 1999). The gene mutated in *pandora* is *spt6* and morpholino-mediated knockdown recapitulates the mutant phenotype (Keegan et al., 2002). The results from the *mil*MO and *cas*MO embryos suggest that the origin of the PEO lies within the LPM. In order to further test this hypothesis, I assayed *wt1* and *tcf21* gene expression on *spt6*MO

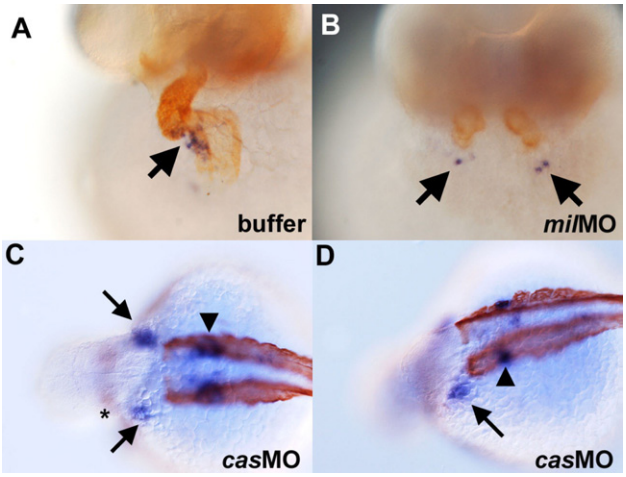

Fig. 5. Bifid hearts are each associated with their own proepicardial organ. Face views of buffer-injected (A) and *mil*MO-injected (B) embryos stained with MF20 against myosin heavy chain (brown) and *wt1* to mark the PEO (blue). A PEO cluster is visible at near the AV junction in buffer-injected embryos (arrow in panel A) while each heart in the *mil*MO embryo is associated with a cluster of *wt1*-positive PEO (arrows in panel B). (C) Dorsal and (D) dorsolateral views on embryos injected with *cas*MO. The nephric *wt1* expression is indicated by the arrowheads and lies ventral to the anterior somites stained with MF20 (brown). Large, diffuse clusters of *wt1* cells can be seen in the lateral positions (arrows). Dorsal views are shown because these cells fail to reach their more ventral positions next to the heart, seen here below the plane of focus in brown (asterisk). Anterior is to the left in panels C and D.

embryos. The PEO in these embryos is not visible using both *wt1* and *tcf21* as molecular markers (Fig. 6). Visualization of myosin heavy chain using the MF20 antibody does indicate a smaller heart with a stalk-like ventricle as previously described (Yelon et al., 1999).

Cell polarity complexes are required for proper PEO development

Other heart morphology mutants have been described including a class with enlarged hearts, typified by the *heart of glass* (*heg*) mutation (Stainier et al., 1996). *heg* encodes a transmembrane protein required for the concentric growth of the myocardium (Mably et al., 2003). Morpholino knockdown of *heg*, however, leads to an enlarged heart as assayed by MF20 staining but a relatively normal PEO as assayed by both *wt1* and *tcf21* expression (Figs. 6D, H, I). I also tested whether heart function is required for proper PEO development since hemodynamic forces have been shown to play a role in organ morphogenesis of the kidney and heart (Hove et al., 2003; Serluca et al., 2002). Knockdown of *cardiac troponin T*, the gene mutated in *silent heart*, does not affect the development of the PEO (Figs. 6J–L).

The *heart and soul* (*has*) mutation disrupts cardiac chamber development. Mutant hearts form two concentric rings with an outer atrial chamber and an inner ventricular mass (Stainier et al., 1996). The genetic lesion in *has* lies in the gene encoding atypical protein kinase C (aPKC) or protein kinase C iota (PRKCI), a gene known to be involved in determining cell polarity (Horne-Badovinac et al., 2001; Peterson et al., 2001). Morpholino antisense oligonucleotide was used to knockdown *has* function and recapitulate the mutant phenotype (Fig. 7A). Curiously, *has*MO embryos display an unexpected PEO phenotype. Staining with *wt1* indicates a failure of the PEO progenitors to reach their proper position (Fig. 7B). The *wt1*-positive cells are found on either side of the midline heart suggesting that the migration of the progenitor cells requires *has/aPKC/PRKCI* gene function (115/186, 62%). A scattered pattern can also be observed in other *has*MO embryos where the *wt1*-positive cells do not form clear bilateral foci but are distributed along multiple vectors (Fig. 7C, 61/186, 33%).

has/PRKCI/aPKC is part of a larger complex of proteins, the Par3–Par6–aPKC complex which localizes to the apical membrane in polarized epithelia and functions to establish and maintain cell polarity (Suzuki and Ohno, 2006). A second complex, the crumbs–stardust/PALS1–DiscsLost/PATJ, is also apically localized and serves to establish cell polarity. Components of these complexes have been shown to interact biochemically (Hurd et al., 2003; Wang et al., 2004). To test whether the PEO migration defect of *has*MO embryos is due to

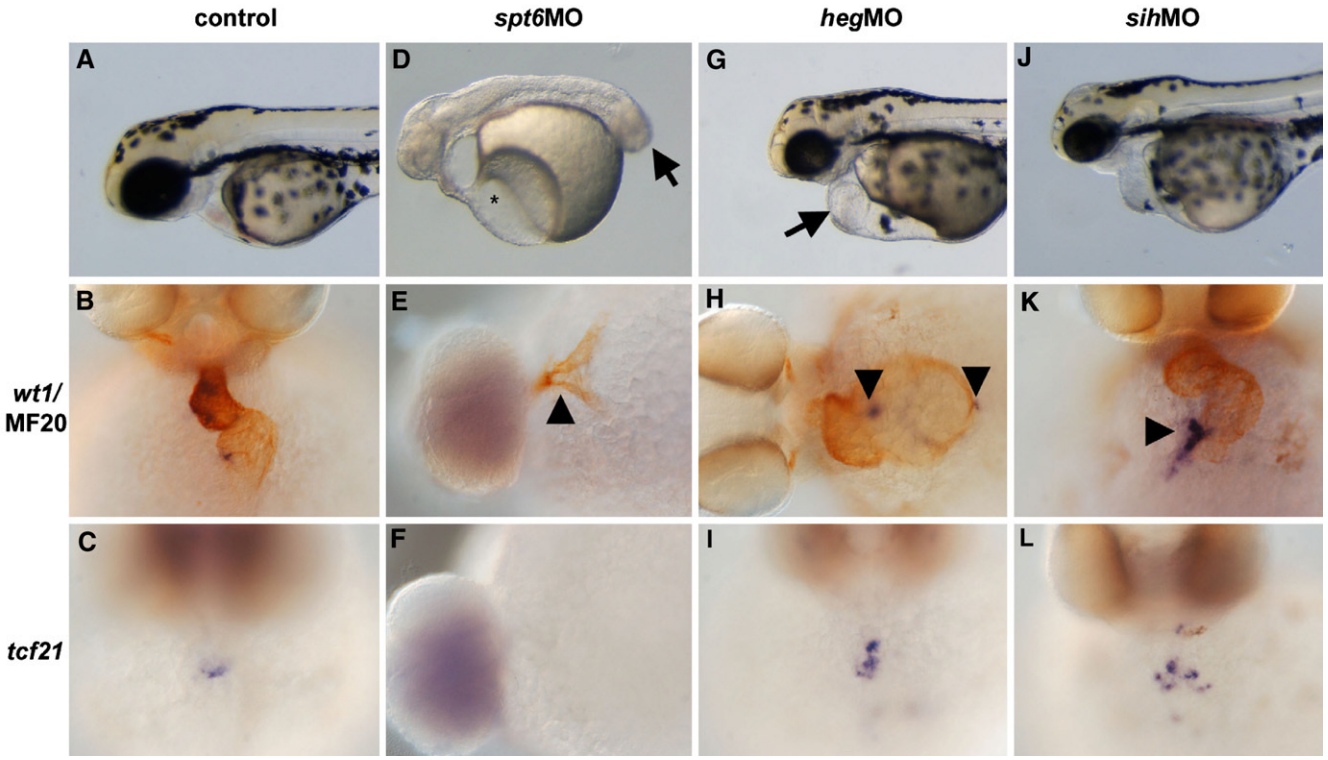

Fig. 6. *pandora*/*spt6* is required for the PEO lineage. (A) Buffer-injected control embryos shown as a live 48 hpf embryo, (B) stained with *wt1* (blue)/MF20 (brown) to mark the PEO and myocardium respectively, and with *tcf21* (C) as a second marker of the PEO. (D) *spt6*MO embryos shown as live 48 hpf embryos. Note the extensive pericardial edema (asterisk) and tail extension defects (arrow). The PEO is not visible either by (E) *wt1* or (F) *tcf21* staining. Myocardium is still present, however, as evidenced by the MF20 positive tissue in the *wt1*/MF20 double-stained *spt6*MO embryos (arrowhead in panel E). (G) *heg*MO embryos display an enlarged heart (arrow) but relatively a relatively normal PEO as assayed by (H) *wt1*, with two *wt1*-positive clusters at the characteristic positions (arrowheads) and (I) *tcf21* expression. (J) *sih*MO embryos shown live at 48 hpf. Staining with (K) *wt1* and (L) *tcf21* reveals no effect of heart function on the formation of the PEO (arrowhead in panel K). Anterior is to the left in all panels except B, C, I, K and L which are face views with dorsal at the top of the panel.

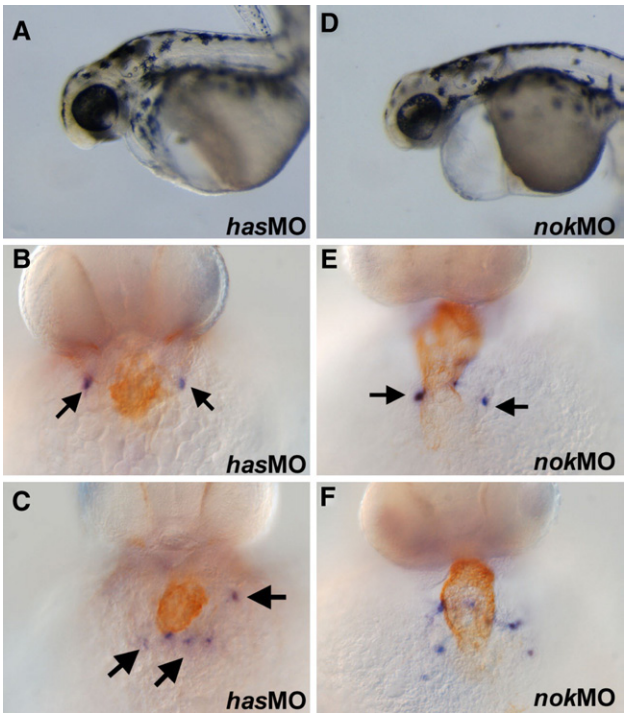

Fig. 7. The cell polarity genes *has* and *nok* are required for proper PEO formation. (A) *has*MO embryos shown live at 48 hpf. (B) *has*MO embryo stained with MF20 (brown) and *wt1* (blue) displays a midline heart but bilateral clusters of *wt1* cells (arrows). (C) An example of a *has*MO embryo displaying scattered *wt1*-positive cells (arrows) adjacent to the heart. (D) 48 hpf embryos injected with *nok*MO displaying characteristic pericardial edema. (E and F) *nok*MO embryo at 48 hpf stained with *wt1* (blue) and MF20 (brown). Note the midline heart in but the bilateral spots of *wt1* expression (arrows in panel E) and scattered *wt1*-positive cells in panel F.

a disruption of this cell polarity machinery, I used a morpholino antisense to knockdown *nagie oko* (*nok*), a functional zebrafish homolog of the *Drosophila stardust* gene (Rohr et al., 2006). Knockdown of *nok* gene function in embryos results in a similar PEO defect as seen in *has*MO embryos where *wt1*-positive PEO cells are scattered and located in more lateral positions (Figs. 7D–F). As with *has*MO embryos, *wt1*-positive cells can be found in either a bilateral (26/122, 21%) or scattered arrangement (93/122, 76%). In contrast to *has*MO, most of the embryos display the scattered arrangement.

Discussion

The thickening and vascularization of the myocardial wall and the presence of valves to ensure unidirectional flow are essential in generating the higher blood pressures of vertebrate organisms. Both characteristics require an intact epicardial layer to provide appropriate signaling cues. The epicardium develops from the PEO and relatively little is known about the mechanisms guiding its development in comparison to myocardium and endocardium development and differentiation. This study introduces a genetic framework outlining the early development of the PEO by specifically examining its development in embryonic phenotypes originally identified by genetic screens for cardiac morphology and function.

Development of the PEO

The transcription factor *wt1* is expressed in the epicardium of mammals and birds and is also expressed in teleosts (Armstrong et al., 1993; Carmona et al., 2001; Moore et al., 1999). Examination of *wt1* expression during the early steps of epicardial development allows for the tracking of its progenitor cells. The nature of the *wt1*-positive cells and PEO was confirmed by testing with another second maker, *tcf21*. The *tbx5* gene, which is also expressed in the PEO of multiple species, is not expressed in a pattern similar to that of *wt1* or *tcf21* in the zebrafish (Garrity et al., 2002).

Expression of *wt1* is first observed at 40 hpf and remains relatively unchanged when examined at 48 hpf. *wt1*-positive cells contact the heart near its atrioventricular junction or the sinus venosus as it does in other model systems. *wt1* expression at later stages suggests that cells migrate anteriorly along the dorsal surface of the heart (Fig. 3E), and then eventually cover the heart by 4 dpf. The punctate nature of the *wt1* staining at day three of development suggests that the migration of the epicardium over the hearts surface may not occur as an epithelial sheet as it does in avian species. Direct cell labeling experiments or observation of a transgenic animal with a reporter protein driven by the *wt1* or *tcf21* promoters would be required to fully verify this hypothesis.

Most of the cardiac morphology mutations described in the original large-scale screens have been well characterized and the mutated genes identified. However, the characterization was limited to the myocardium and endocardium as no tools were available to mark the epicardium or the PEO. Since it was not known where the PEO progenitors lie nor whether the heart tube is necessary for induction of the PEO, it was not clear whether the PEO would develop in association with each bilateral heart or independently at the midline in mutants displaying a cardia bifida phenotype. In other model systems, the PEO appears as a mesothelial outgrowth near the transverse septum and there is no obvious link in the origins of myocardial and epicardial cells and thus one might predict that only one PEO would form in bifid mutants although recent data indicate the presence of two PEO anlagen at early stages in both the mouse and chick (Schulte et al., 2007). However, unlike the mouse, PEO markers (*wt1*) are never seen in the bilateral state during normal zebrafish development.

The formation of two PEOs in *mil*MO embryos suggests that either the migration defect underlying the bifid heart phenotype may also affect the migration of epicardial progenitors or the heart tube can induce the PEO fate in adjacent tissues. Given the observation that the *wt1*-positive clusters in *cas*MO do not lie in close proximity to the myocardium, as they do in *mil*MO embryos, it is likely that the earliest known epicardial progenitors lie in the lateral plate mesoderm. The appearance of *wt1*-positive cells in the *mil*MO and *cas*MO is also different. The *wt1*-positive area in *cas*MO appears larger, diffuse and is not in close proximity to the bifid hearts, in contrast to the relatively normal PEOs found in *mil*MO embryos. It is possible that *casanova* function or endoderm is required not only for the migration of the PEO

progenitors from lateral positions but for the proper morphogenesis of the proepicardial organ itself.

In both the *mil*MO and *cas*MO cases, the anterior LPM is prevented from midline migration, via distinct mechanisms, and two PEOs result strongly suggesting an LPM origin. The observation that each of the bilateral hearts in these embryos is associated with an area of *wt1*-positive cells strongly suggests an LPM origin of the PEO. Further evidence for a LPM origin of the PEO comes from the *spt6*MO embryos which contain fewer *cmlc2*- and *vmhc*-positive progenitor cells in the LPM and lack the PEO entirely. Direct evidence such as lineage marking of cells would be required to formally determine its origin with the LPM.

Conserved role for wt1 in epicardium development

Knockdown of *wt1* in zebrafish embryos indicates that function as well as expression of the transcription factor is conserved in teleosts since no *tcf21*-positive cells are visible in *wt1*MO embryos. At later stages, however, a fraction of the embryos display *tcf21*-positive cells adjacent to the myocardium. It is possible that the *wt1*MO is diluted out at these later stages due to cell divisions and that the remaining wild-type message (Supplemental Figs. 1 and 3) is sufficient to allow PEO development to proceed in some embryos. Quantitative RT-PCR results do not support this notion since the level of knockdown appears to be the same at 2 and 4 days post-fertilization. It remains possible that *wt1* may only be required for proper timing of PEO development. Alternatively, the observation that patches of epicardium form at the caudal end of the ventricles in *WT1* null mice also suggests mechanisms for epicardial development that are independent of *wt1* gene function. In contrast to the mouse knockout, heart chamber morphology is not affected in *wt1*MO embryos, at least up to 4 dpf. However, at this stage, the epicardium has just fully covered the myocardium and signaling roles for the epicardium, as observed in other species, cannot be excluded from occurring at later time points.

Cell polarity and PEO morphogenesis

Two categories of PEO phenotypes are observed in knockdowns of the cell polarity genes *has*/*aPKC*/*PRKCI* and *nagie oko*. In one (bilateral), the *wt1*-positive cells are found on either side of the midline heart tissue (Figs. 7B, E), in the other, the *wt1*-positive cells are found adjacent to the heart but distributed along many directions and do not form clear bilateral foci (Figs. 7C, F). It is difficult to determine using *in situ* hybridization, whether the underlying cause is due to a migration defect, a clustering defect or both. Whatever the principal cause may be, the morphogenesis of the PEO is clearly disrupted.

In one possible model, the cell polarity of the PEO cells or the cells with which they come into contact is necessary for directed migration toward the midline. This is in contrast to its role in cardiac chamber differentiation since myocardial and endocardial precursors do reach the midline in *has* and *nok* mutants (Horne-Badovinac et al., 2001; Peterson et al., 2001; Rohr et al., 2006). Thus, a failure to migrate may translate into a bilateral phenotype whereas misguided migration may manifest itself as scattered *wt1*-positive cells. A role for the cell polarity machinery in cell migration has been demonstrated in astrocytes. Par6 and aPKC both localize to the leading edge of migrating astrocytes along with CDC42 (Etienne-Manneville and Hall, 2001). In this system, aPKC has been shown to phosphorylate GSK3β leading to a polarization of the microtubule organizing center and thus direct the path of cell protrusions (Etienne-Manneville and Hall, 2003). It is tempting to speculate that a similar mechanism may be used by migrating epicardium progenitor cells in the developing embryo.

A second polarity complex, the crumbs–stardust/PALS1–DiscsLost/PATJ complex, is also involved in establishing cell polarity and directly interacts with the PAR6 protein via stardust/PALS1 (Hurd et al., 2003). A role for this second complex in cell migration has not been described as it has for the PAR3–PAR6–aPKC complex. Knockdown of a zebrafish *stardust* homolog, *nagie oko*, yields a similar result to that observed in *has*MO albeit with a distinct distribution of bilateral and scattered phenotypes. Thus while it is clear that both cell polarity complexes are required for proper morphogenesis, this distinction suggests that the two complexes do not function in an identical fashion.

A role for the cell polarity gene *Par3* in murine epicardium development has also been described (Hirose et al., 2006). Par3 is required for epicardial precursor cyst formation but migration toward the myocardium is not disrupted. This appears to be a distinct phenotype from the *has*MO and *nok*MO phenotypes observed in fish and may reflect species specific differences in epicardial development. In the chick, the epicardial precursors generally cover the heart as a continuous epithelial sheet whereas in mice, isolated patches of epicardial sheets are seen and this last method requires *Par3* gene function. It is not clear from this first study which method is preferentially used in teleosts.

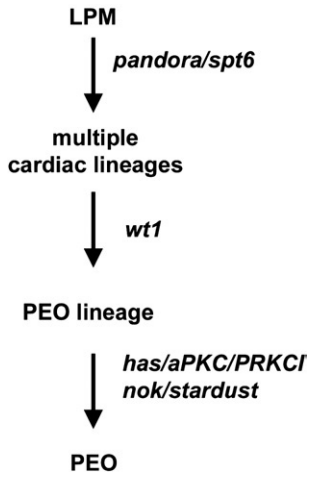

Fig. 8. A stepwise model for PEO development. The *pandora/spt6* gene is required to specify multiple cardiac lineages including the epicardium from lateral plate mesoderm progenitors. The *wt1* gene is required for the development of the PEO lineage only. The cell polarity genes *has/aPKC/PRKCI* and *nok/stardust* are not required for the differentiation of PEO cells but rather the morphogenesis of the organ.

Steps in PEO development

The results from the gene knockdown experiments described here can be formulated into a genetic framework where *pan/spt6* is required at the earliest steps to specify multiple cardiac lineages, *wt1* is required for the epicardial lineage only and the cell polarity genes *has/aPKC/PRKCI* and *nok/stardust* are required for proper migration toward the midline and morphogenesis of the PEO (Fig. 8). While both *spt6*MO and *wt1*MO embryos lack PEO marker expression, it is reasonable to place *spt6* upstream of *wt1* for two reasons. First, *spt6* is expressed at a much earlier time point and the transcript is maternally stored (Keegan et al., 2002). In contrast, *wt1* expression first appears during early somitogenesis and this in nephrogenic mesoderm (Serluca and Fishman, 2001). Second, *spt6* is required for the epicardium and myocardial lineages while *wt1* is only required for the epicardium. Following lineage specification, morphogenesis of the PEO requires the cell polarity machinery to either direct cell migration from lateral positions to the midline or for proper clustering of the PEO or both.

It has also been proposed recently that the epicardium can control the regulation of the heart's regenerative potential (Lepilina et al., 2006). Zebrafish have a capacity to regenerate several tissues including the heart (Poss et al., 2002). This regenerative potential is either absent or lies dormant in mammals and comparisons of mammal and teleost epicardial layers may further elucidate the source of this regenerative potential. The use of *wt1* or *tcf21* as a probe in zebrafish would allow the identification of mutations and pathways that selectively perturb the epicardium. These would be of particular interest because of the epicardium's potential for regenerative signals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.10.007.

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